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The bio-mineralization of virus-like particles by metal-organic framework nanoparticles enhances the thermostability and immune responses of the vaccine

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Experimental

Reagents and antibodies

Zinc nitrate hexahydrate ($Zn(NO_3)_2.6H_2O$) (80141318), 2-methylimidazole (693-98-1), and lipopolysaccharide (LPS) (0000089171) were purchased from Sigma-Aldrich. Bovine serum albumin (BSA) was purchased from VWR. 3-(4,5-dimethylthiazol-2-yl)-5-(3- carboxymethoxyphenyl) -2-(4-sulfophenyl)-2H-tetrazolium (MTS) was obtained from Promega. Thiophene- 2-thiol, 4,6-Dia-midino-2-phenylindole (DAPI) was obtained from Beyotime. Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin (PS), and fetal bovine serum (FBS) were purchased from Gibco. Anti-CD3e-FITC (0023186), anti-CD4-APC (0143017), and anti-CD8a-PE (0198990) were bought from BD Biosciences. FITC-conjugated goat anti-pig IgG (F4762), HRP-conjugated anti-guinea pig IgG (A60-110P), and β-mercaptoethanol were purchased from Sigma-Aldrich. Interleukin-4 (IL-4, 404-ML-050/CF), granulocyte-macrophage colony-stimulating factor (GM-CSF, 415-ML-050/CF), and the ELISA kits for tumor necrosis factor-α (TNF-α, MTA00B), interleukin-6 (IL-6, M6000B), interleukin-12p70 (IL-12p70, P247617) and interferon-γ (IFN-γ,

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P193773) were purchased from R&D Systems. Micro BCA Protein Assay Kit (23235) was obtained from Thermo Fisher Scientific. Anti-FMDV polyclonal antibodies produced from pig, rabbit, and guinea pig were provided by the OIE/China National Foot-and-Mouth Disease Reference Laboratory. All other reagents were of analytical grade and used as received. Millipore Milli-Q water (>18 MΩ•cm) was used for all solutions.

Characterization of nanoparticles

The characterization of nanoparticles was performed by transmission electron microscopy (TEM; HT7700, Hitachi, Japan) operated at 80 kV, Fourier Transform Infrared Spectra (FTIR; Nicolet 360, USA), and dynamic light scattering (DLS; Malvern Zetasizer-Nano ZS90, Britain). X-ray measurements were performed on a Bruker D8 FOCUS Powder X-ray Diffractometer using Cu Kα radiation. The operation voltage and current were kept at 40 kV and 40 mA.

Cells

Baby hamster kidney (BHK-21) cells, Porcine kidney (PK-15) cells, RAW 264.7 cells were cultured in DMEM medium supplemented with 10% FBS and 1% penicillin-streptomycin (PS) at 37℃ in a humidified atmosphere containing 5% CO_2.

The influence of 2-methylimidazole on the reactogenicity of VLPs

VLPs were incubated with different concentrations of 2-methylimidazole solution (500 mM, 250 mM, 125 mM, 62.5 mM, 31.25 mM, 15.62 mM，7.81mM, 3.9 mM) for the same time as mineralization. The reactogenicity of VLPs was measured by ELISA.

The stability of VLPs-ZIF-8 in different media

The VLPs-ZIF-8 nanoparticles were placed in different solutions of deionized water, normal saline, DMEM, and simulated body fluid (SBF). The samples were collected at different time periods. The collected samples were measured by dot blotting to detect antigen release by VLPs-ZIF-8.

Isolation of DCs

The bone-marrow-derived dendritic cells (BMDCs) were isolated from BALB/c mice fed in specific-pathogen-free conditions. Briefly, the bone marrow was collected and lysed in red blood lysis buffer. Then the cells were collected by centrifugation (1000 rpm, 10 min) and cultured in RPMI 1640 medium with 10% FBS, 1% PS, 20 ng mL^{-1} granulocyte-macrophage colony-stimulating factor (GM-CSF), 20 ng mL^{-1} interleukin 4 (IL-4), and 0.02‰ β -mercaptoethanol at 37 °C under 5% CO_2. After

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incubation for 12 h, the unattached cells were discarded, and the fresh medium was added. The medium was changed every two days. The cells were observed under a microscope after 5-days cultures.

Cellular uptake of VLPs-ZIF-8

Indirect immunofluorescence: BHK-21 cells seeded on coverslips were incubated with VLPs or VLPs-ZIF-8 containing the same amount of protein (5 μg mL^{-1}) when grown to 60% confluence. After incubation for different periods (0.5 h, 2 h, and 5 h) in a cell incubator, the cell samples were fixed with 4% paraformaldehyde for 15 min then treated with 0.1% Triton X-100 for 15 min at RT. Next, the cell samples were sequentially incubated with pig anti-FMDV polyclonal antibody (1:200) and FITC-conjugated goat anti-pig IgG (1:100) for 1 h at 37°C, then stained with DAPI for 15 min at RT. The cells were washed with PBST three times after each treatment. Finally, the samples were observed using laser confocal microscopy (LSCM, Leica SP8, Leica Inc, Solms, Germany) at excitation wavelengths of 488 and 405 nm, and the representative images were captured.

Western blotting: RAW 264.7 and BHK-21 cells were seeded on a 12-well plate in 1 mL of culture medium. After growing to 80% confluence, the cells were incubated with VLPs or VLPs-ZIF-8, containing identical doses of VLPs (5 μg mL^{-1}) for different times (1, 2, 4, and 6 h). The treated cells were washed with PBS solution, and the total cell samples were collected. Then the samples were separated by SDS-PAGE and transferred to an NC membrane. After blocking with 5% skim milk solution, incubation with FMDV primary antibody and HRP-secondary antibody, the target protein band was visualized through a Luminous fluid.

Endo-lysosomal escape

Endo-lysosomal escape was observed through laser confocal microscopy. BHK-21 cells with 50%~60% confluence were incubated with VLPs-ZIF-8 for 0.5 h, 2 h, and 5 h, respectively. Then the cells were treated with Lyso-Tracker Red solution for 1 h in the dark after being washed with PBS and followed by the procedure of indirect immunofluorescence to visualize VLPs-ZIF-8 and nuclei. The stained cells were observed under the laser confocal microscopy (Leica SP8, Leica Inc, Solms, Germany).

Biochemical indexes evaluation

Blood was collected from mice in different immunization groups (VLPs group, VLPs-ZIF-8 group, VLPs-ISA206 group), and serum was separated. The biochemical indexes (aspartate aminotransferase, creatine kinase, creatinine, and total bilirubin) were measured to determine the influence of vaccines used in

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different immunization groups on the biochemical levels. The biocompatibility of different vaccines was further determined by biochemical indicators.

The biodistribution of VLPs-ZIF-8

The VLPs-ZIF-8 was injected into mice through the tail vein, and the uninjected mice were used as blank control. After 48 h, the mice were sacrificed and the main organs (heart, liver, spleen, lung, kidney) of VLPs-ZIF-8–injected mice and blank mice were collected. The content of zinc in each organ was detected by ICP-MS. The biodistribution of VLPs-ZIF-8 was determined by comparing the zinc content in each organ with the blank group.

Animal experiment

All animal procedures were performed following the Guidelines for Care and Use of Laboratory Animals of Lanzhou Veterinary Research Institute, Chinese Academy of Agriculture Sciences, and approved by the Animal Ethics Committee of Lanzhou Veterinary Research Institute and the Chinese Academy of Agriculture Sciences (approval number: LVRIAEC2018-008).

A total of forty-two healthy female BALB/c mice (6～7 week-old) were randomly divided into seven groups (n = 6) to detect the adjuvant effect of ZIF-8 and the thermostability of VLPs. These mice were immunized with sterile PBS (control group), VLPs (50 μg, 4 ^{o}C), VLPs (50 μg)-ZIF-8 (4 ^{o}C), VLPs (50 μg)-ISA206 (4 ^{o}C) , VLPs (50 μg, stored at 37 ^{o}C for 7 days), VLPs (50 μg)-ZIF-8 (stored at 37 ^{o}C for 7 days), VLPs (50 μg)-ISA206 (stored at 37 ^{o}C for 7 days) via intramuscular injection at multiple points, respectively. The mice blood at 28 days after immunization was collected to measure the antibody levels. The mice were weighed on the 0th day, 14th day, and 28th day to evaluate the biosecurity.

Histological examination

The immunized mice were sacrificed after 28 days, and the main organs (heart, liver, spleen, lung, and kidney) were collected and immersed in 4% paraformaldehyde. Then the organs were embedded in paraffin, cut into 4 μm slides, and stained with hematoxylin and eosin (H&E). Then the slides were observed and imaged under a digital trinocular microscope (BA400Digital).

Cytokines secretion in RAW264.7 and dendritic cells

RAW 264.7 cells (1×10^5 cells mL^{-1}) and immature DCs (1×10^6 cells mL^{-1}) were seeded in 12-well plates in 1 mL of culture medium, respectively. The cells were treated with PBS, ZIF-8, VLPs, VLPs-ZIF-8, and LPS, respectively. After incubation for 12 h, the supernatant

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was collected. According to the manufacturer's instructions, the cytokines, IL-6 and TNF-α from RAW 264.7 cells, IL-12p70 from DCs were detected using a mouse ELISA kit.

Specific antibody levels

The specific antibody level of mice sera was tested by liquid blocking ELISA (LB-ELISA). Briefly, 96-well plates were coated with rabbit anti-FMDV antibody diluted with 1000 folds at 4℃ overnight. The sera were serially diluted and incubated with an equivalent volume of inactivated FMDV virus at 4℃ overnight. The positive serum, negative serum, and antigen virus were set as controls. After washing with PBST, the coated 96-well plates were added of above antigen-antibody composite and incubated at 37℃ for 1 h. Then the plates were washed with PBST three times, and guinea pig anti-FMDV antibodies were added and incubated for 1 h. Then the plates were washed with PBST three times, the HRP-anti-guinea pig antibody was added and incubated for 1 h. Then the plates were washed with PBST three times, the enzyme-substrate was added. Then the absorbance was measured with a microplate reader (Bio-Rad, USA) after the reaction was stopped with stop buffer H_2SO_4 (2 M).

IgG1 and IgG2a antibody levels

The mice sera were collected 28 days after immunization to detect total IgG1 and IgG2a levels. In brief, anti-mouse IgG1 and IgG2a antibodies were diluted 1000 folds with PBS solution, added to 96-well plates at 100 μL well^{-1} respectively, and incubated for 1 h at 37 ^{o}C. The coating solution was removed, and the plates were washed three times with washing buffer. The sera samples diluted with PBS solution were added to the coated plates at 100 μL well^{-1} and incubated at RT for 1 h. After washing three times with washing buffer, the diluted goat anti-mouse antibody was added to 96-well plates at 100 μL well^{-1} and incubated at RT for 0.5 h. After washing three times with washing buffer, the diluted peroxidase-labeled rabbit anti-goat antibody was added to 96-well plates at 100 μL well^{-1} and incubated at RT for 0.5 h. After washing three times with washing buffer, the substrate solution was added to the 96-well plates. With incubation for 15 min, the absorbance was measured with a microplate reader (Bio-Rad, USA), with the reaction was stopped with stop buffer H_2SO_4 (2 M).

Lymphocyte proliferation

The spleens of mice were collected 28 days after immunization. The spleens were ground and lysed in red blood cell lysis buffer. After being washed with sterile PBS, the cells were suspended in RPMI 1640

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medium containing 10% FBS and 1% PS. Then the cells were counted and seeded in a 96-well plate with 1×10^5 cells $well^{-1}$. The culture medium group was used as the blank group, and the untreated lymphocyte group was served as the negative control group. The VLPs (10 μg mL^{-1}) was added to stimulate the lymphocytes as the VLPs group. Three replicates were set for each sample. The MTS reagent (10 μL $well^{-1}$) was added to each well after incubation for 68 h. Then the absorbance at 490 nm was recorded through a microplate reader (Bio-Rad, USA).

$$Proliferation\ rate\ (\%) = \frac{\overline{A}(\text{VLP group}) - \overline{A}(\text{blank group})}{\overline{A}(\text{negative control group}) - \overline{A}(\text{blank group})}$$

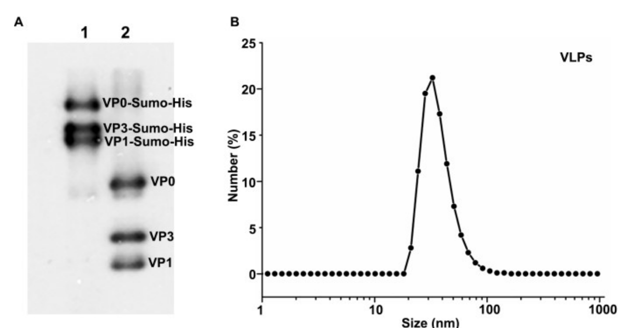

Fig. S1: Characterization of FMDV-VLPs. (A) Western blotting of purified proteins and digested proteins. Lane 1: His-SUMO tagged-capsid proteins of FMDV; Lane 2: Enzyme-digested capsid proteins without His-SUMO tag. (B) DLS measured the hydrated particle size of FMDV-VLPs.

Cytokines of the splenocytes and activation of CD4$^+$ T and CD8$^+$ T Cells

The harvested spleen lymphocytes were stimulated with VLPs and incubated for 24 h. The supernatants were used to test cytokines of IFN-γ, IL-4, TNF-α and IL-6 using an ELISA kit according to the manufacturer's instructions. The cells were resuspended in PBS containing 2% FBS and 1% PS and incubated with fluorescent antibodies, anti-CD3e-FITC, anti-CD4-APC, and anti-CD8a-PE at 4℃ for 0.5～1 h. After washing with PBS twice, the labeled cells were resuspended in PBS containing 2% FBS and 1% PS and analyzed by flow cytometry (BD, USA)

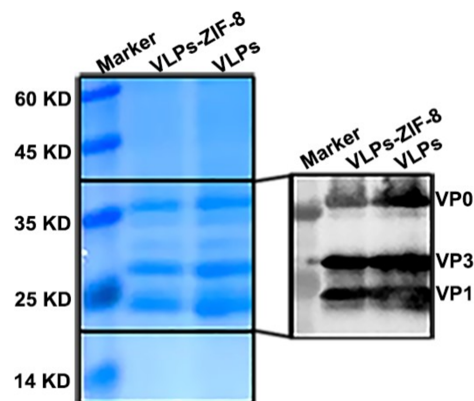

Fig.S2: Identification of VLPs-ZIF-8 complex. The complex of VLPs-ZIF-8 was identified by SDS-PAGE and western blotting.

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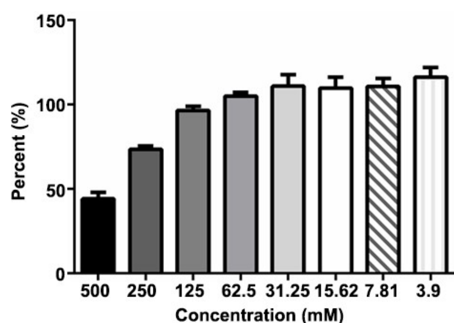

Fig.S3: Influence of 2-methylimidazole on VLPs reactivity. The influence of different concentration of 2-methylimidazole on VLPs reactivity were measured by ELISA.

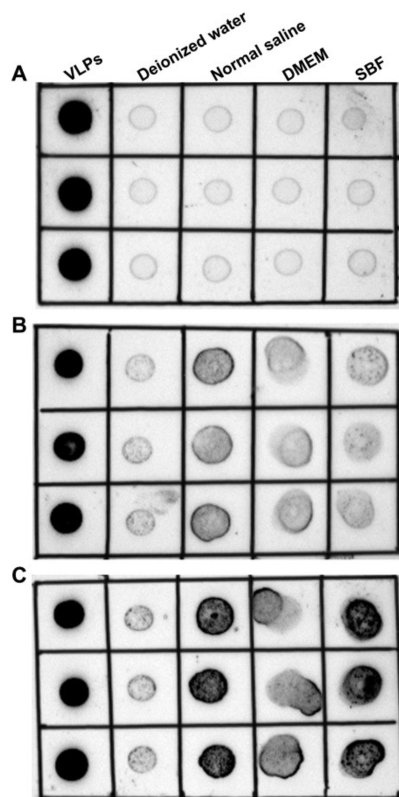

Fig.S4: The stability of VLPs-ZIF-8 in different media. The levels of antigen released by the VLPs-ZIF-8 complex in different solutions at different time periods (A:0 h, B: 12 h, C：24 h) were detected by dot blot.

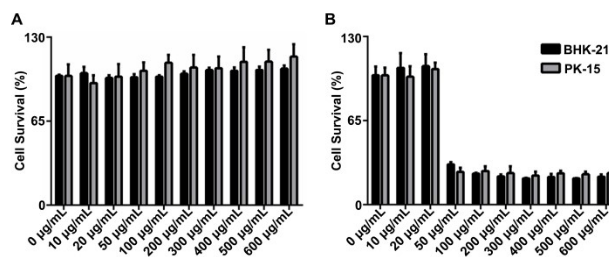

Fig. S5: The cytotoxicity of Zn $(NO_3)_2$•$6H_2O$ and 2-methylimidazole. The cytotoxicity of different concentrations of 2-methylimidazole (A) or Zn $(NO_3)_2$•$6H_2O$ (B) to BHK-21 cells and PK-15 cells was measured by MTS.

Table S1 The biochemical indexes

Group	Biochemical molecules	Detection value	Reference
VLPs	Aspartate aminotransferase	138.7	60.0-220.0
	Creatine kinase	841.2	5.0-1000.0
	Creatinine	25.6	22.0-97.0
	Total bilirubin	14.94	2.00-20.00
VLPs-ZIF-8	Aspartate aminotransferase	98.2	60.0-220.0
	Creatine kinase	443.7	5.0-1000.0
	Creatinine	24.3	22.0-97.0
	Total bilirubin	11.05	2.00-20.00
VLPs-ISA206	Aspartate aminotransferase	104.0	60.0-220.0
	Creatine kinase	396.4	5.0-1000.0
	Creatinine	23.4	22.0-97.0
	Total bilirubin	13.40	2.00-20.00

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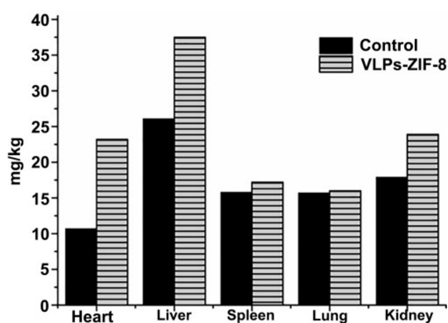

Fig.S6: The biodistribution of VLPs-ZIF-8. The distribution of VLPs-ZIF-8 complexes was measured by ICP-MS determination of zinc ion content in different organs.

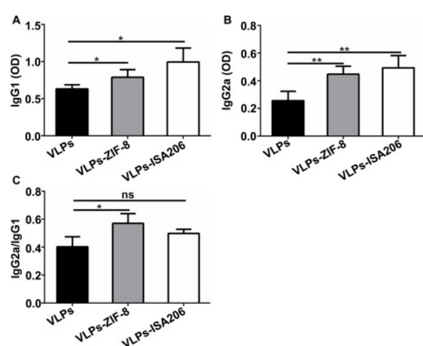

Fig. S7: IgG1 and IgG2a antibody levels. Levels of IgG1 (A) and IgG2a (B) in mice sera were detected by indirect ELISA. (C) The calculated ratio of IgG2a/IgG1.

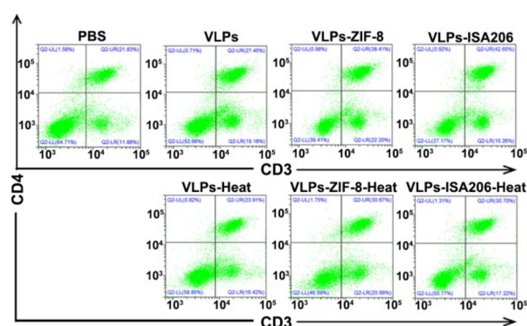

Fig.S8: $CD4^+$ T lymphocyte levels were determined by flow cytometry.

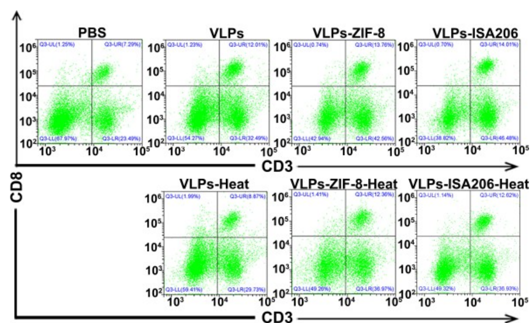

Fig.S9: $CD8^+$ T lymphocyte levels were determined by flow cytometry.

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